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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
The overall objective of this project is to investigate the molecular mechanisms of Nip3's function in apoptosis and the role of Nip3 in chemoresponses of breast cancer cells. The first aim is to determine whether Nip3 acts directly or indirectly to induce cell death. The results showed that Nip3 does not directly induce cell death in yeast. Next, the role of Bax in Nip3 action was evaluated in the induction of yeast cell death. When Bax and Nip3 were co-transfected into yeast cells, no synergy between Bax and Nip3 in cell death induction was observed. Co-immunoprecipitation experiments also showed that there is no direct interaction between Bax and Nip3. Thus Nip3 induces apoptosis independent on Bax but requires additional factors. The potential Nip3 interacting proteins that collaborate with Nip3 to induce cell death in yeast is currently being investigated by yeast two-hybrid cloning. A parallel study of other BH3 domain containing protein has resulted the cloning of a new member of the Bcl-2 family, Bcl-G. Over-expression of Bcl-G_L or Bcl-G_S in cells induced apoptosis, but Bcl-G_S was far more potent than Bcl-G_L. Apoptosis induction by Bcl-G_S depended on the BH3 domain, and was suppressed by co-expression of anti-apoptotic Bcl-X_L protein. Bcl-G gene is located in chromosome 12p12, a region frequently subjected to loss of heterozygosity in cancer patients. The role of Bcl-G in breast cancer development and chemoresponse will be further studied.

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Introduction

The overall objective of this project is to investigate the molecular mechanisms of Nip3's function in apoptosis and the role of Nip3 in chemoresponses of breast cancer cells. The first aim is to determine whether Nip3 acts directly or indirectly to induce cell death. Next, the role of Bax in Nip3 action was evaluated in the induction of yeast cell death. The potential Nip3 interacting proteins that collaborate with Nip3 to induce cell death in yeast will be identified by yeast two-hybrid cloning. Studies are also in progress to address the mechanisms of Nip3 activation in breast cancer cells during responses to chemotherapeutic drugs. A parallel study aims to clone a new member of the Bcl-2 family, Bcl-G. Bcl-G gene is located in chromosome 12p12, a region frequently subjected to loss of heterozygosity in cancer patients. The role of Bcl-G in breast cancer development and chemoresponse will be further studied.

Annual report

Part 1. To investigate the molecular mechanisms of Nip3's function in apoptosis (month 1-20).

Task 1: Determine whether Nip3 induces apoptosis directly or indirectly by studies in yeast (month 1-2).
Nip3 cDNA was constructed into pESC-His yeast expression vector at BamHI/XhoI sites. The empty control pESC vector and pESC/Nip3 were transfected into EGY48 yeast cells, using standard yeast transfection protocol. The transfectant cells were cultured in BMM plates containing galactose to activate Nip3 expression. As shown in Figure 1, Nip3 does not induce cell death in yeast cells.

Task 2: If Nip3 acts directly, determine the channel forming activity of Nip3, induction of cytochrome c release by Nip3, and effects of Nip3 on mitochondrial membrane potential (months 3-8); mutagenesis study of the role of Nip3 dimerization in apoptosis (months 9-14); complementation cloning of proteins required for Nip3-induced apoptosis (15-20).
Task 1 results indicate that Nip3 does not induce cell death directly.

Task 3: If Nip3 acts indirectly, determine the role of Bax in Nip3-induced apoptosis and cytochrome c release, the effects of Nip3 on Bax channel activity and oligomerization state (months 3-10).
To study the role of Bax in Nip3's function, human Bax cDNA was constructed into YEp51 yeast expression vector. YEp51/Bax and pESC/Nip3 were transfected alone or together into EGY48 yeast cells. As shown in Figure 2, when grown on galactose containing plates, Bax induced significant level of yeast cell death. There was no synergy observed in induction of cell death when Nip3 and Bax were co-expressed in yeast. To test whether Bax interacts with Nip3, Nip3 cDNA was constructed into a pcDNA3 vector with T7 epitope tag. T7-Nip3 and Bax were cotransfected into 293T cells. The lysates were co-immunoprecipitated with T7 antibody or a polyclonal anti-Bax antibody. The samples were then blotted with Bax or T7 antibody, respectively. As shown in Figure 3, there is no interaction detected between Bax and Nip3. Interestingly, in the western blot study of Nip3 expression, Nip3 was observed to be expressed as a dimer in mammalian 293T cells, but expressed as a monomer in yeast cells (Figure 4). This difference may account for the failure to induce cell death by Nip3 in yeast cells. To test this possibility, Nip3 gene will be constructed into FK506 binding protein vector to express FKBP-Nip3. We can then use FK1012 to force Nip3 dimerization in yeast cells. If dimerized Nip3 can induce cell death in yeast, the experiments proposed in task 2 will be performed to study mechanisms of Nip3-induced cell death.

Task 4: Identify proteins that interact with Nip3 by two-hybrid cloning and split ubiquitin cloning (months 11-20).
Nip3 cDNA was constructed into pGilda yeast two-hybrid vector. Using pGilda-Nip3 as bait, a human testis cDNA library was used in yeast two-hybrid screening to screen for potential Nip3 interacting proteins. Briefly, pGilda-Nip3 and a reporter plasmid pSH18-34 were co-transfected into EGY48 yeast cells. The tranfectant yeast cells were then

transfected in large scale with yeast two-hybrid library cDNA plasmids. Positive clones were identified through selection in Leucine deficient media. A total of 155 positive colonies were identified. These positive clones were further examined for a second reporter expression by β-gal filter assays. Among these clones, 59 of them were tested positive. These clones will be further examined by mating assays to confirm the interaction with Nip3. Plasmids from the positive clones will be isolated and sequenced to reveal their identity. The interaction between candidate gene and Nip3 will then be confirmed by co-immunoprecipitation studies.

Part 2. To investigate the role of Nip3 in the responses to chemotherapy by breast cancer cells (months 21-36).

Task 5: Determine the changes of Nip3 protein in breast cancer cells during drug treatment and the associated mechanisms (months 21-23); determine the role of Nip3 in drug-induced apoptosis, cytochrome c release and downstream mechanisms in breast cancer cells by gene transfection and antisense oligonucleotides (months 24-27); determine drug-induced Nip3 activation in terms of phosphorylation and association with cytosolic proteins (months 28-32).
In an initial study to examine the changes of Nip3 protein level during response to chemotherapy by breast cancer cells, MCF-7 and PC-3 cells were treated with Doxorubicin or VP-16. Nip3 protein level was examined by western blot using a poly-clonal anti-Nip3 antibody. As shown in Figure 5, there was no change of Nip3 protein level after drug treatment.

Part 3. In a parallel study, a novel gene in the Bcl-2 family was cloned and its function was studied. For details of this study, please see the appended publication.

A new member of the Bcl-2 family was identified, Bcl-G. The human *BCL-G* gene consists of 6 exons, resides on chromosome 12p12, and encodes two proteins through alternative mRNA splicing: Bcl-G (long) and Bcl-G (short) consisting of 327 and 252 (length) amino-acids, respectively. Bcl-G_L and Bcl-G_S are identical in their first 226 amino-acids but diverge thereafter. Among the Bcl-2 Homology (BH) domains previously recognized in Bcl-2 family proteins, the BH3 domain is found in both Bcl-G_L and Bcl-G_S, but only the longer Bcl-G_L protein possesses a BH2 domain. Bcl-G_L mRNA is expressed widely in adult human tissues, whereas Bcl-G_S mRNA was found only in testis. Over-expression of Bcl-G_L or Bcl-G_S in cells induced apoptosis, but Bcl-G_S was far more potent than Bcl-G_L. Apoptosis induction by Bcl-G_S depended on the BH3 domain, and was suppressed by co-expression of anti-apoptotic Bcl-X_L protein. Bcl-X_L also co-immunoprecipitated with Bcl-G_S but not with mutants of Bcl-G_S in which the BH3 domain was deleted or mutated and not with Bcl-G_L. Bcl-G_S was predominantly localized to cytosolic organelles whereas Bcl-G_L was diffusely distributed throughout the cytosol. A mutant of Bcl-G_L in which the BH2 domain was deleted displayed increased apoptotic activity and co-immunoprecipitated with Bcl-X_L, suggesting that the BH2 domain auto-represses Bcl-G_L.

Key Research Accomplishments

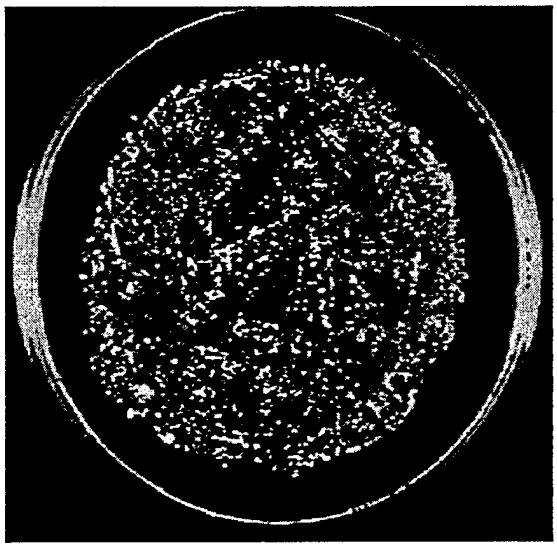
1. Nip3 does not induce cell death in yeast cells, indicating that Nip3 requires other protein(s) to act together in cell death induction.
2. Bax does not interact with Nip3, nor does it function together with Nip3 in inducing cell death.
3. In yeast cells, Nip3 is expressed as monomer. In contrast, Nip3 is a dimer in 293T cells.
4. Potential Nip3 interacting proteins were identified through yeast two-hybrid screening. Their role in Nip3 dimerization, activation and cell death induction will be further examined.
5. A novel member of the Bcl-2 family, Bcl-G, was cloned and its function in regulation of apoptosis was studied. This study has resulted a publication in Journal of Biological Chemistry.

Reportable Outcomes

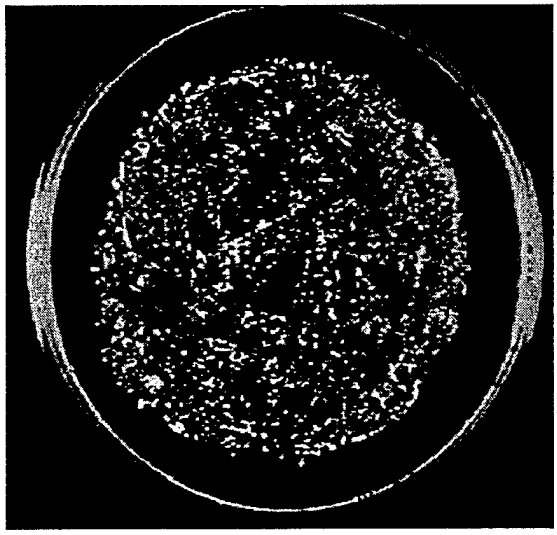
1. A publication on Journal of Biological Chemistry.
   Guo B, Godzik A, Reed JC. 2001 Bcl-G, a novel pro-apoptotic member of the Bcl-2 family. *J. Biol. Chem.* 276(4): 2780-5.

Conclusions

1. Nip3 does not induce cell death by itself. Proteins other than Bax are required to function together with Nip3 to induce cell death.
2. Yeast two-hybrid screening assay will reveal the protein factors that interact with Nip3, and provide important information on the mechanisms of Nip3 induced cell death.
3. A novel BH3 domain containing protein, Bcl-G, was identified. Bcl-G interacts with Bcl-XL and promotes apoptosis. Loss of heterozygosity of Bcl-G gene was frequently found in cancer patients. Bcl-G is a potential candidate for the tumor suppressor located on chromosome 12p12. Studies of Bcl-G mutation and its regulation of apoptosis in breast cancer will provide important information on breast cancer development and improve chemoresponse.



pESC



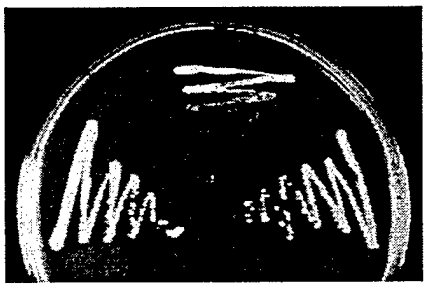
pESC/Nip3

Fig. 1

A

Bax

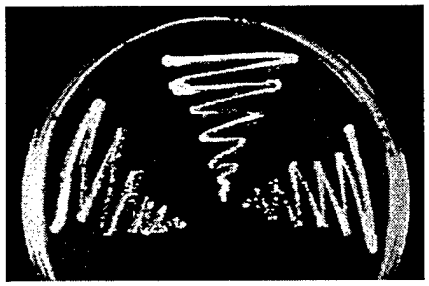
YEp51



Bax+
Nip3

Nip3

pESC

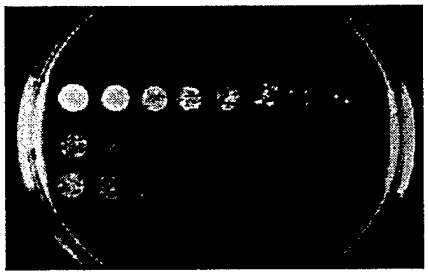


Bax+
Nip3

B

10^4 $5x10^3$ 10^3 500 100 50 10 5

Yep51
Bax
Bax+
Nip3



pESC
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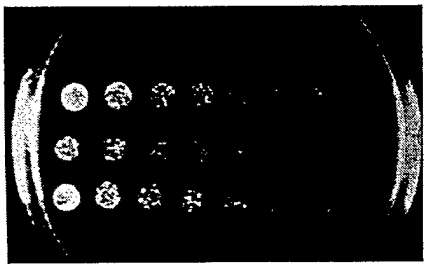

Fig. 2

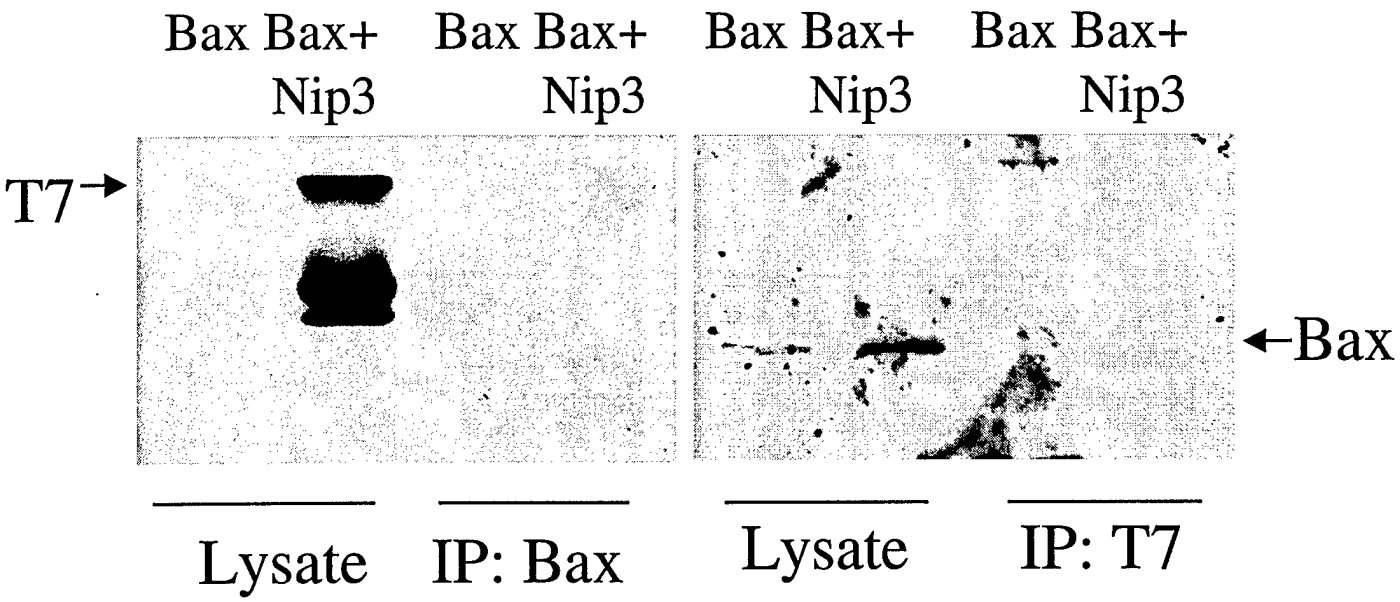
Bax Bax+ Nip3
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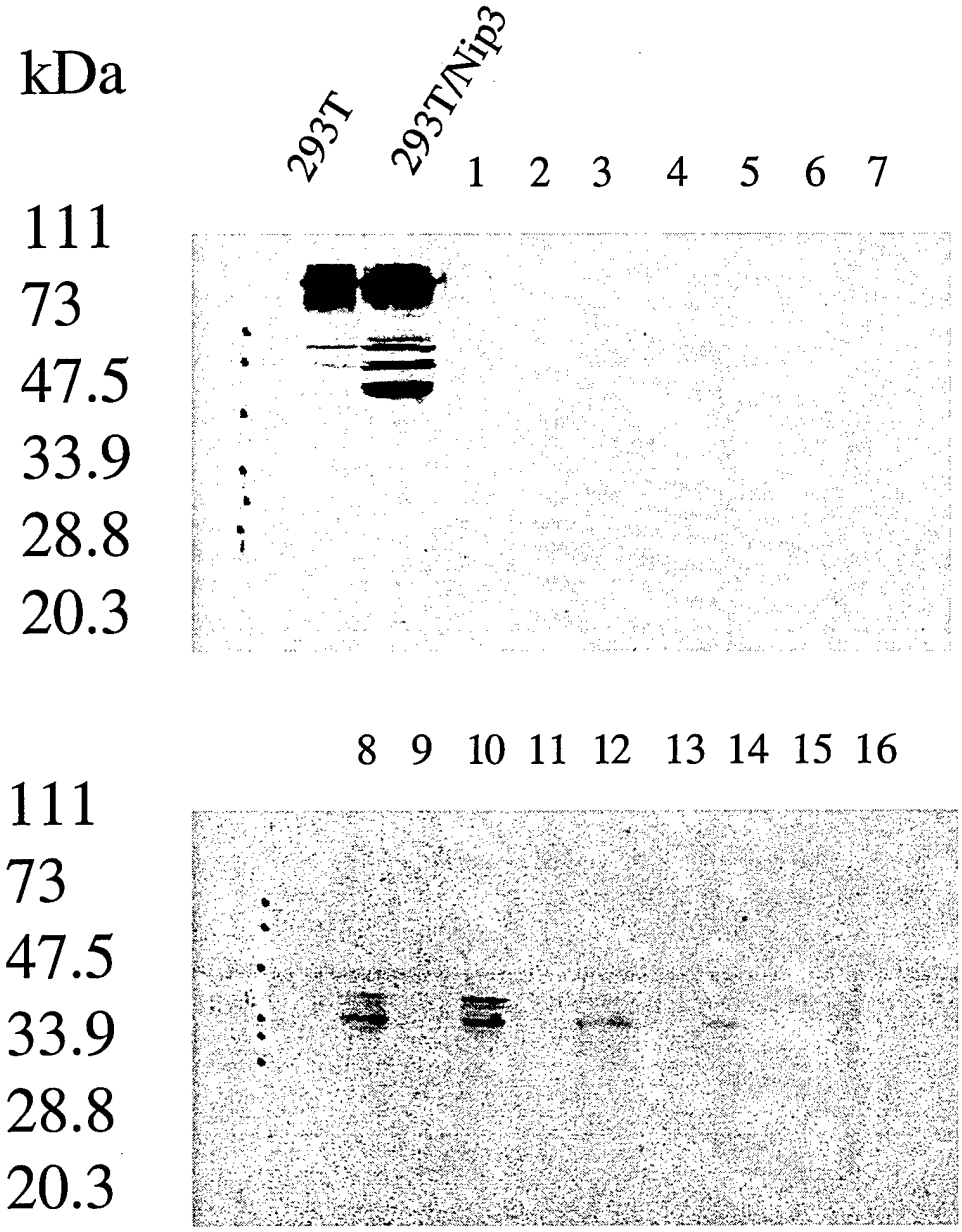
Fig. 3


kDa
293T
293T/Nip3
1 2 3 4 5 6 7
111
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47.5
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Fig. 4

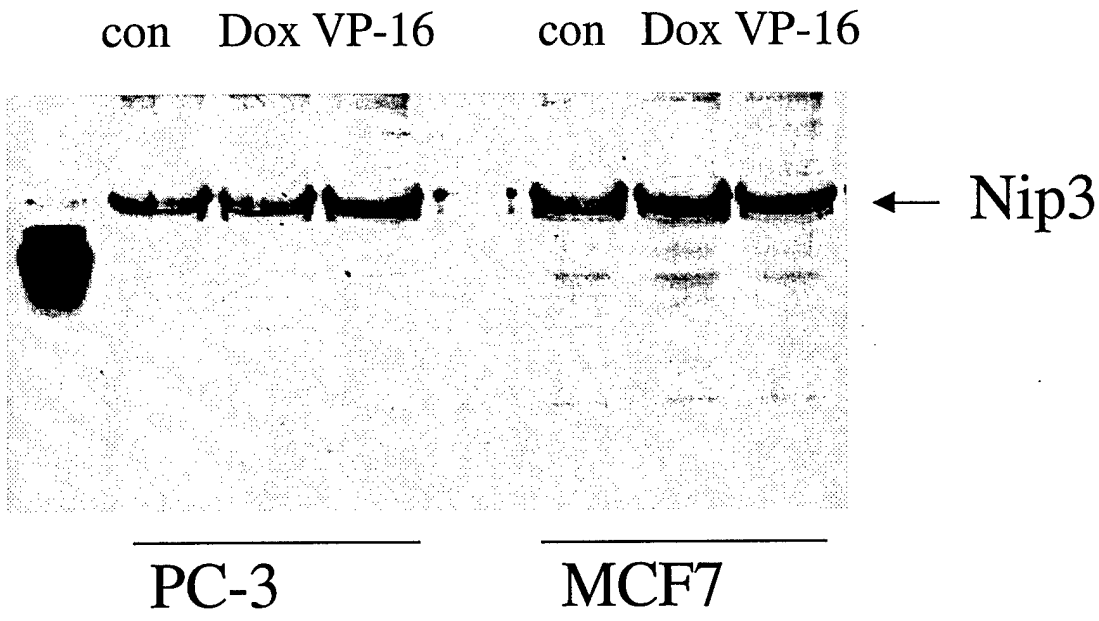

Fig. 5

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Bcl-G, a Novel Pro-apoptotic Member of the Bcl-2 Family*

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A new member of the Bcl-2 family was identified, Bcl-G. The human *BCL-G* gene consists of 6 exons, resides on chromosome 12p12, and encodes two proteins through alternative mRNA splicing, Bcl-G_L (long) and Bcl-G_S (short) consisting of 327 and 252 amino acids in length, respectively. Bcl-G_L and Bcl-G_S have identical sequences for the first 226 amino acids but diverge thereafter. Among the Bcl-2 homology (BH) domains previously recognized in Bcl-2 family proteins, the BH3 domain is found in both Bcl-G_L and Bcl-G_S, but only the longer Bcl-G_L protein possesses a BH2 domain. Bcl-G_L mRNA is expressed widely in adult human tissues, whereas Bcl-G_S mRNA was found only in testis. Overexpression of Bcl-G_L or Bcl-G_S in cells induced apoptosis although Bcl-G_S was far more potent than Bcl-G_L. Apoptosis induction by Bcl-G_S depended on the BH3 domain and was suppressed by coexpression of anti-apoptotic Bcl-X_L protein. Bcl-X_L also coimmunoprecipitated with Bcl-G_S but not with mutants of Bcl-G_S in which the BH3 domain was deleted or mutated or with Bcl-G_L. Bcl-G_S was predominantly localized to cytosolic organelles, whereas Bcl-G_L was diffusely distributed throughout the cytosol. A mutant of Bcl-G_L in which the BH2 domain was deleted displayed increased apoptotic activity and coimmunoprecipitated with Bcl-X_L, suggesting that the BH2 domain autorepresses Bcl-G_L.

Bcl-2 family proteins are central regulators of apoptosis (reviewed in Refs. 1–3). Bcl-2 family proteins are conserved throughout the animal kingdom with homologs identified in both vertebrates and invertebrates. These proteins contain up to four conserved Bcl-2 homology (BH)[1] domains, BH1, BH2, BH3, and BH4, which are recognized by their amino acid sequence similarity. Both anti- and pro-apoptotic Bcl-2 family proteins have been identified. These proteins control cell life/death decisions through their effects on events such as mitochondrial release of proteins involved in activation of caspase-family cell death proteases or by binding sequestering caspase-activating proteins (reviewed in Refs. 2–5 and 7–8). Many Bcl-2 family proteins are capable of physically interacting with each other, forming a complex network of homo- and heterodimers, and these physical interactions sometimes play important roles in the opposing effects of pro- and anti-apoptotic members of the family.

The pro-apoptotic members of the Bcl-2 family can be broadly classified into two groups. One group, including Bax, Bak, and Bok in humans, shares structural similarity with the pore-forming domains of certain bacterial toxins and is capable of forming pores in synthetic membranes *in vitro* (9–12). These proteins exhibit cytotoxic effects independently of their ability to bind other Bcl-2 family proteins including Bcl-2 and other cytoprotective members of the family such as Bcl-X_L, Bcl-W, Bfl-1, and Mcl-1. The second group of pro-apoptotic Bcl-2 family proteins varies widely in their amino acid sequences, often containing only a single region of similarity, specifically, the BH3 domain. These "BH3-only" proteins appear to possess no intrinsic or autonomous cytodestructive activity and instead operate as *trans*-dominant inhibitors of the survival proteins. Their antagonism of proteins such as Bcl-2 and Bcl-X_L depends on binding via their BH3 domains to a hydrophobic pocket on target anti-apoptotic proteins (13).

Gene knockout studies in mice have demonstrated nonredundant roles for various Bcl-2 family genes in regulating cell life and death in specific tissues or under particular physiological or pathological circumstances (14–18). Thus, it is important to identify all members of the Bcl-2 family and to delineate the cellular contexts in which they contribute to apoptosis regulation. In this report, we have described the cloning and initial characterization of a new member of the Bcl-2 family, Bcl-G.

MATERIALS AND METHODS

Cloning of Bcl-G cDNAs—TBLAST searches of the public databases using human Bcl-2 as a query sequence revealed a short EST (GenBank™/EBI no. AW000827) from colonic mucosa of 3 patients with Crohn's disease, which contains an open reading frame (ORF) encoding sequences similar to the BH2 domain of Bcl-2 family proteins. An oligonucleotide primer (5′-GTACTTGGTGCCAAAGCCCAGG-3′) was designed complementary to the EST sequence and used for 5′-RACE, employing the SMART™RACE cDNA Amplification Kit (CLONTECH) and human placental total RNA as template. The 5′-RACE products were subcloned into pCR2.1-TOPO vector using the TOPO™ TA Cloning kit (Invitrogen), and the DNA sequence was determined revealing a complete ORF with start codon within a favorable Kozak sequence context preceded by a 5′-UTR containing stop codons in all three reading frames (GenBank™/EBI accession nos. AF281254 and AF281255). Two additional EST clones, AI478889 and AI240211, were identified by BLAST searches and correspond to overlapping partial Bcl-G cDNAs that contain the 3′-UTR. BLAST searches of GenBank™/EBI also revealed a 190,858 base pair human BAC clone (RPCI11–267J23) in the genomic database derived from chromosome 12p12 (GenBank™/EBI no. AC007537), which contains the *BCL-G* gene in its entirety.

Plasmids—cDNAs containing the ORFs of Bcl-G_L and Bcl-G_s without additional flanking sequences were generated by PCR using human placental cDNA as a template and the following primers: 5′-GGCTCG-AGCGATGTGTAGCACCAGTGGGTGTGACC-3′, sense for both Bcl-G_L

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF281254, AF281255.

‡ Recipient of a Postdoctoral Traineeship Award from the United States Army MRMC Breast Cancer Research Program.

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[1] The abbreviations used are: BH, Bcl-2 homology domain; EST, expressed sequence tag; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; GFP, green fluorescent protein; PKA, cAMP-dependent kinase; PKC, protein kinase C; UTR, untranslated region.

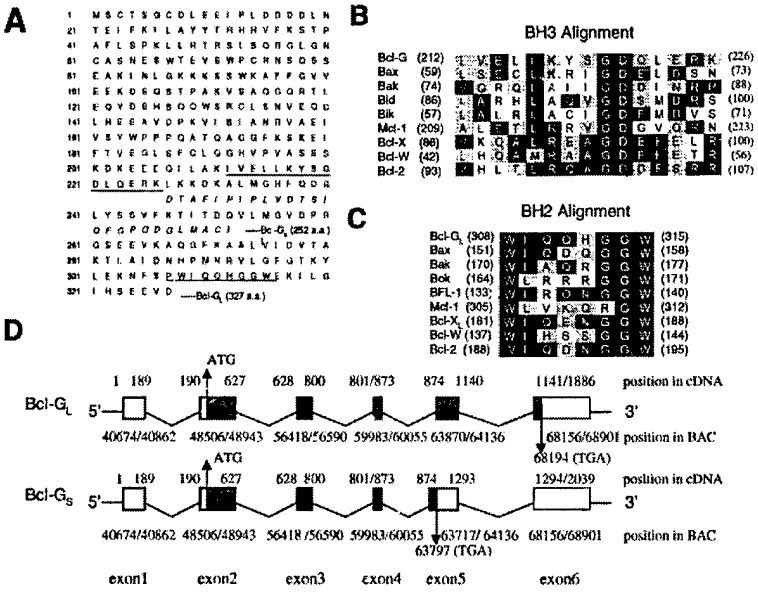

FIG. 1. **Sequence analysis of Bcl-G cDNAs.** *A*, the predicted amino acid sequences of the Bcl-G_L and Bcl-G_s proteins are presented with the BH2 and BH3 domains *underlined* and residue numbers indicated. The predicted proteins are identical from residues 1–226. The unique C-terminal region of Bcl-G_S is indicated in *italics*. *B*, an alignment is presented of the BH3 domains of Bcl-G_L and several other Bcl-2 family proteins. Identical and similar residues are shown in *black* and *gray blocks*, respectively. *C*, an alignment is presented of the BH2 domains in Bcl-G and several other Bcl-2 family proteins, as above. *D*, the exon-intron organization of the *BCL-G* gene is presented. The human *BCL-G* gene contains 6 exons, spanning a ~30 kilobase region of chromosome 12. Alternative splicing at the 5′-end of exon 5 accounts for the production of the Bcl-G_L and Bcl-G_S proteins, where splice-acceptor sites at nucleotide positions 63,870 *versus* 63,797 in BAC clone RPCI 11–267J23 (GenBank™/EBI no. AC007537) are utilized for Bcl-G_L and Bcl-G_S, respectively. The positions of the start and termination codons are indicated, with coding regions in *gray blocks* and noncoding 5′- and 3′-UTR sequence in *open blocks*. The BH3 domain is located in exon 4 of both Bcl-G_L and Bcl-G_S, whereas the BH2 domain resides in exon 5 of Bcl-G_L.

and Bcl-G_s; 5′-CCAAGCTTTAAGTCTACTTCTTCATGTGATATCCC-3′, antisense for Bcl-G_L; 5′-CCAAGCTTTAAAATGCAGGCCATCAAA-CC-3′, antisense for Bl-G_S. The resulting PCR products were digested with restriction endonucleases and subcloned into the *Xho*I and *Hin*dIII sites of pEGFP-C1 (CLONTECH). A mutant of Bcl-G_S lacking the BH3 domain was created by a two-step PCR method, using the following primers: primer1, 5′-GGCTCGAGCGATGTGTAGCACCAGTGGGTGT-GACC-3′; primer2, 5′-CCGGATCCGGCTAGTATTTGTTCTTCTTCAT-CTTTC-3′; primer3, 5′-CCGGATCCGACACTGCCTTCATCCCCATTC-CC-3′; and primer4, 5′-CCAAGCTTTAAAATGCAGGCCATCAAACC-3′. The resulting PCR product was digested with *Xho*I/*Bam*HI or with *Bam*HI/*Hin*dIII respectively and ligated into pEGFP-C1. Site-directed mutagenesis of Bcl-G_s was performed to generate a L216E substitution mutation using the QuikChange™ Site-directed Mutagenesis kit (Stratagene) following the manufacturer's protocol, with pEGFP-C1/Bcl-G_s plasmid as DNA template and the mutagenic primers: 5′-GCC-AAAATTGTTGAGCTGGAGAAATATTCAGGAGATCAGTTGG-3′ and 5′-CCAACTGATCTCCTGAATATTTCTCCAGCTCAACAATTTTGGC-3′. A mutant of Bcl-G_L lacking the BH2 domain was created by PCR using the same forward primer for Bcl-G_L and 5′-GGAAGCTTCA-GAAGTTCTCTTTCAGGTACTTGG as the reverse primer.

Measurements of Bcl-G mRNAs—Bcl-G mRNAs were detected by either Northern blotting or RT-PCR. For RT-PCR, we employed multiple tissue cDNA panels (CLONTECH) containing first-strand cDNA generated from 16 different tissues. PCR was performed according to the manufacturer's protocol with the following primers: (*a*) 5′-primer for both Bcl-G_s and Bcl-G_L, corresponding to exon 3, 5′-CTGAGGGTCTC-TCCTTCCAGCTCCAAGG-3′; (*b*) 3′-primer for Bcl-G_L, corresponding to exon 5, 5′-GGCCGTGACGTCTATTACAAGGGCAGCC-3′; 3′-primer for Bcl-G_s, corresponding to an alternatively spliced segment of exon 5, 5′-CAAGGGAATGGGGATGAAGGCAGTGTC-3′. Human glyceraldehyde-3-phosphate dehydrogenase expression was examined by PCR with the following primers: sense, 5′-TGAAGGTCGGAGTCAACGGAT-TTGGT-3′; antisense, 5′-CATGTGGGCCATGAGGTCCACCAC-3′.

Cell Culture, Transfections, and Apoptosis Assay—293T and COS-7 cells were cultured in Dulbecco's modified Eagle's high glucose medium (Irvine Scientific, Santa Ana, CA) containing 10% fetal bovine serum. PC-3 cells were cultured with RPMI 1640 media containing 10% fetal bovine serum. Transfection of cells was performed using SuperFect (Qiagen, Chatsworth, CA). Both floating and adherent cells (after trypsinization) were collected 24 h after transfection, fixed, and stained using 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) for assessing apoptosis based on nuclear fragmentation and chromatin condensation (19, 20).

Coimmunoprecipitations and Immunoblotting—Immunoblotting was performed as described previously (19, 20). For coimmunoprecipitations, cells were cultured in 50 mM benzocarbonyl-Val-Ala- Asp-fluoromethylketone (zVAD-fmk) to prevent apoptosis. Cells were suspended in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 20 mM EDTA, 50 mM NaF, 0.5% Nonidet P-40, 0.1 mM Na_3VO_4, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). Lysates (0.2 ml diluted into 1-ml final volume of lysis buffer) were cleared by incubation with 15 ml of protein G-Sepharose 4B (Zymed Laboratories Inc.) and then incubated with 15 μl of polyclonal anti-GFP antibody (Santa Cruz Biotechnology) and 15 μl of protein G at 4 °C overnight. Beads were then washed four times with 1.5 ml of lysis buffer before boiling in Laemmli sample buffer and performing SDS-polyacrylamide gel electrophoresis and immunoblotting.

Confocal Microscopy—GFP-expressing cells were incubated with 50 nM Mitotracker Red CMXRos (Molecular Probes) for 30 min at 37 °C in culture medium. The cells were then washed with phosphate-buffered saline, fixed in 4% formaldehyde, and imaged by confocal microscopy using a Bio-Rad MRC 1024 instrument (19, 21).

RESULTS

Identification and Sequence Analysis of the BCL-G gene and cDNAs—A short EST was identified during searches of the public databases, which when conceptually translated revealed a polypeptide sequence with similarity to the BH2 domain of Bcl-2 family proteins. Full-length cDNAs were obtained revealing two potential transcripts containing ORFs for proteins of 327 and 252 amino acids, respectively, which we have termed Bcl-G_L and Bcl-G_S (Fig. 1A). The predicted Bcl-G_L and Bcl-G_S proteins are identical for the first 226 amino acids and then diverge thereafter. Comparison of the predicted amino acid sequences of Bcl-G_L and Bcl-G_S with Bcl-2 family proteins revealed the presence of a candidate BH3 domain in both Bcl-G_L and Bcl-G_S (Fig. 1, *A* and *B*) and the presence of a BH2

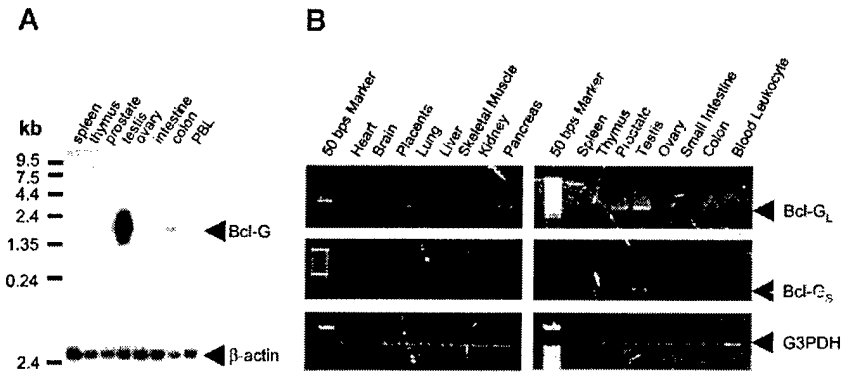

FIG. 2. **Expression of Bcl-G_s and Bcl-G_L in human tissues.** The expression of transcripts encoding Bcl-G_L or Bcl-G_S was examined by Northern blotting (*A*) and RT-PCR (*B*). For Northern blotting, poly(A)$^+$-selected RNA samples from various adult tissues were analyzed by hybridizing the blot with a ^{32}P-labeled Bcl-G_L cDNA probe. The blot was stripped and reprobed with a β-actin cDNA (*bottom*). Molecular mass markers are indicated in kilobase pairs. For RT-PCR, first-strand cDNA prepared using RNA samples from various adult human tissues was PCR amplified using primers specific for Bcl-G_L and Bcl-G_S based on differences in splice-acceptor utilization in exon 5. The primers flank an intron in both cases, thus excluding amplification because of contaminating genomic DNA. PCR products were size-fractionated in 2% agarose gels, stained with ethidium bromide, and then photographed under UV-illumination.

domain in Bcl-G_L but not in Bcl-G_S (Fig. 1, *A* and *C*). Using the Bcl-G cDNA sequences, the human genomic database was searched, revealing a BAC clone from chromosome 12p12 containing the *BCL-G* gene. Comparison with the cDNA sequences suggests a 6-exon structure for the *BCL-G* gene. The Bcl-G_L and Bcl-G_S cDNAs can be accounted for by an alternative mRNA splicing mechanism in which different splice acceptor sites associated with exon 5 are employed, resulting in a change in the distal reading frame (Fig. 1*D*).

Tissue-specific Expression of Bcl-G_L and Bcl-G_S mRNAs—Northern blotting demonstrated the presence of Bcl-G transcripts of ~1.5–2.5 kilobase pairs in length in several adult human tissues (Fig. 2*A*), but the highest levels of Bcl-G mRNA by far were found in male gonad (testis), thus prompting the moniker "Bcl-Gonad" (Bcl-G). Because Northern blotting failed to resolve the mRNAs encoding Bcl-G_L and Bcl-G_S, we designed RT-PCR assays using primers specific for Bcl-G_L and Bcl-G_S sequences associated with exon 5. The amplified bands corresponding to Bcl-G_L and Bcl-G_S were excised and sequenced, confirming the validity of the RT-PCR strategy (not shown). Bcl-G_L mRNA was clearly detected in lung, pancreas, prostate, and testis, with lower levels present in some other tissues (Fig. 2*B*). In contrast, Bcl-G_S mRNA was uniquely expressed in testis. RT-PCR amplification of a control mRNA, glyceraldehyde-3-phosphate dehydrogenase, demonstrated loading of nearly equivalent amounts of mRNA from each tissue.

Induction of Apoptosis by Bcl-G Proteins—To assess the effects of Bcl-G_L and Bcl-G_S on apoptosis, various cell lines, including COS-7, HEK293T, and PC3, were transiently transfected with plasmids encoding Bcl-G_L or Bcl-G_S. For most experiments, Bcl-G_L and Bcl-G_S were expressed as GFP fusion proteins so that successfully transfected cells could be conveniently identified (Fig. 3*A*), but similar results were obtained when FLAG-epitope tags were employed instead (not shown). Overexpression of the shorter Bcl-G_S protein reproducibly induced striking increases in the percentage of cells undergoing apoptosis, as determined by DAPI staining (Fig. 3*A*) and other methods (not shown). In contrast, Bcl-G_L was more variable and less efficient at inducing apoptosis in these transient transfection assays. Immunoblot analysis of lysates from transfected cells demonstrated that the less potent effects of Bcl-G_L could not be accounted for by lower levels of protein production (Fig. 3*A*). Indeed, Bcl-G_L protein accumulated to levels ~10-fold higher in cells compared with Bcl-G_S, suggesting that Bcl-G_S is a far more potent apoptosis inducer. Analysis of the same blots with an anti-tubulin antibody confirmed loading of essentially equivalent amounts of total protein for each sample, thus validating the results. In additional transfection experiments, Bcl-G_L failed to demonstrate cytoprotective activity in side by side comparisons with Bcl-2 and Bcl-X_L (data not presented). Also, when Bcl-G_L was coexpressed with Bcl-G_S in cells, no synergy with or nullification of Bcl-G_S-induced apoptosis was observed.

The BH3 Domain of Bcl-G_S Is Required for Its Pro-apoptotic Activity—The Bcl-G_S protein contains a BH3 domain but lacks other regions of homology with Bcl-2 family proteins. Structural studies indicate that BH3 domains represent amphipathic α-helices in which the hydrophobic surface of the α-helices of apoptosis-inducing BH3 peptides bind to a pocket on survival proteins such as Bcl-X_L (22). We, therefore, compared the apoptosis-inducing activity of the wild-type Bcl-G_S protein with mutants lacking the BH3 domain (ΔBH3) or in which leucine 216 within the BH3 domain of Bcl-G_S was chosen for mutation to the charged glutamic acid, based on comparisons with previously described BH3 mutagenesis experiments demonstrating a critical requirement for the analogous leucine in other pro-apoptotic Bcl-2 family proteins (23, 24).

Wild-type Bcl-G_S potently induced apoptosis when overexpressed in COS-7, PC3, HEK293T, and other cell lines, whereas Bcl-G_S-ΔBH3 and Bcl-G_S-L216E did not (Fig. 3*B* and data not shown). Immunoblot analysis confirmed production of the Bcl-G_S-ΔBH3 and Bcl-G_S-L216E proteins at levels exceeding the amounts of wild-type Bcl-G_S protein. We conclude, therefore, that the BH3 domain of Bcl-G_S is critical for its pro-apoptotic activity.

The BH2 Domain of Bcl-G_L Negatively Regulates Its Pro-apoptotic Activity—Compared with Bcl-G_S, Bcl-G_L only weakly induces apoptosis. Bcl-G_L contains a BH2 domain not found in Bcl-G_S. A mutant of Bcl-G_L lacking the BH2 domain was created. Bcl-G_L-(ΔBH2) induced apoptosis in COS-7 cells as potently as Bcl-G_S (Fig. 3*C*). Thus the BH2 domain negatively regulates the pro-apoptotic activity of Bcl-G_L.

Bcl-G_S Associates with Bcl-X_L in a BH3-dependent Manner—The pro-apoptotic activity of BH3-only members of the Bcl-2 family depends on their ability to dimerize with and suppress the activity of survival proteins such as Bcl-X_L (reviewed in Ref. 13). We, therefore, explored whether Bcl-G_L and Bcl-G_S are capable of associating with other Bcl-2 family proteins by coimmunoprecipitation assays. Bcl-G_S association with the survival proteins Bcl-X_L and Bcl-2 was readily detected by coim-

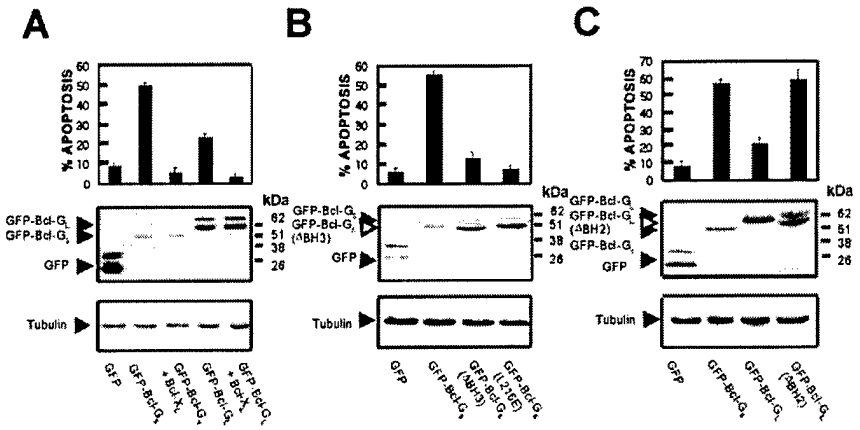

FIG. 3. **Induction of apoptosis by Bcl-G.** *A,* plasmids encoding GFP, GFP·Bcl-G_s, or GFP·Bcl-G_L were transfected into COS-7 cells alone or in combination with a plasmid encoding Bcl-X_L. Apoptosis was examined by DAPI staining at 24 h post-transfection (mean ± S.D., $n = 3$, *top*). Levels of GFP and GFP·Bcl-G fusion proteins were examined by immunoblotting lysates from transfected COS-7 cells (20 μg per lane) and anti-GFP antibody with ECL-based detection (*middle*). Equal loading was confirmed by reprobing the same membrane with anti-tubulin antibody (*bottom*). *B,* plasmids encoding GFP, GFP·Bcl-G_S, or the mutant proteins, Bcl-G_S-ΔBH3 and GFP·Bcl-G_s-L216E were transfected into COS-7 cells. The percentage of apoptotic cells was examined 1 day later as above (*top*). Protein expression was assessed by immunoblotting as above, using anti-GFP (*middle*) or anti-tubulin (*bottom*) antibodies. *C,* plasmids encoding GFP, GFP·Bcl-G_L, or Bcl-G_L-ΔBH2 were transfected into COS-7 cells. The percentage of apoptotic cells was examined 1 day later as above (*top*). Protein expression was assessed by immunoblotting as above, using anti-GFP (*middle*) or anti-tubulin (*bottom*) antibodies.

munoprecipitation using lysates from transiently transfected cells, whereas no association with pro-apoptotic proteins Bax, Bak, Bid, or Bad was observed (Fig. 4*A* and not shown). Interaction of Bcl-G_S with Bcl-2 and Bcl-X_L, but not with Bax or Bak, was also confirmed by yeast two-hybrid assays (not shown). Yeast two-hybrid assays also suggested that no homo- or hetero-dimerization occurred among the Bcl-G_S and Bcl-G_L proteins. In contrast, association of the longer Bcl-G_L protein with Bcl-2 or Bcl-X_L was not easily detected by coimmunoprecipitation assays (Fig. 4*A*). With much longer x-ray film exposure times, however, small amounts of Bcl-X_L were observed in association with Bcl-G_L immunocomplexes, suggesting either low affinity binding of Bcl-G_L to Bcl-X_L or implying that only a small portion of total Bcl-G_L proteins are competent to bind Bcl-X_L (not shown). The interaction of Bcl-G_S with Bcl-X_L was BH3-dependent, as determined by comparisons of wild-type Bcl-G_S with the Bcl-G_S-ΔBH3 and Bcl-G_S-L216E proteins (Fig. 4, *B* and *C*). Thus, the pro-apoptotic activity of Bcl-G_S correlates with it ability to bind Bcl-X_L. When the BH2 domain of Bcl-G_L was deleted, this mutant Bcl-G_L associated with Bcl-X_L (Fig. 4*D*), thus providing further evidence that the BH2 domain autorepresses this protein.

Bcl-G_S Is Associated with Cytosolic Organelles—Many Bcl-2 family proteins, such as Bcl-2, Bcl-X_L, and Bak, contain a hydrophobic stretch of amino acids near their carboxyl terminus that anchors them in intracellular membranes of mitochondria, endoplasmic reticulum, or nuclear envelope (reviewed in Refs. 1–3). However, some pro-apoptotic Bcl-2 family proteins such as Bax, Bid, and Bim are found in the cytosol and must be induced to translocate to mitochondria and other organelles where the Bcl-2-family proteins to which they dimerize reside (25–28). We explored the intracellular locations of the Bcl-G_L and Bcl-G_S protein by two-color confocal microscopy analysis of cells expressing GFP-tagged proteins. GFP·Bcl-G_L protein was located diffusely throughout cells, similar to GFP control protein (Fig. 5). In contrast, Bcl-G_S was found in a punctate cytosolic pattern (Fig. 5), and partially colocalized with a mitochondria-specific dye (Mitotracker). Surprisingly, deletion of the BH3 domain from Bcl-G_S did not disrupt the punctate distribution (Fig. 5), indicating that other regions of the Bcl-G_S protein are sufficient for subcellular targeting. Subcellular fractionation experiments confirmed these observations, demonstrating association of Bcl-G_S and Bcl-G_S-ΔBH3 predominantly with organelle-containing heavy membrane fractions, with scant amounts in the soluble cytosolic compartment (not shown).

DISCUSSION

We describe here a new member of the *BCL-2* gene family in humans, *BCL-G*. The *BCL-G* gene potentially encodes two protein products, Bcl-G_L and Bcl-G_S. Bcl-2 family proteins contain up to four conserved BH domains. The shorter Bcl-G_S protein contains only the BH3 domains, similar to several other pro-apoptotic Bcl-2 family proteins, including Bad, Hrk, Bik, Bim, Blk, Noxa(APR), and Egl1 (reviewed in Refs. 13, 29, 30). In contrast, the longer Bcl-G_L protein contains BH2 and BH3 domains. No other examples of Bcl-2 family proteins are known that combine BH2 and BH3 domains in the absence of BH1. Though the Bad protein was originally suggested to contain a BH2 domain (31) and has been shown to possess the BH3 domain, inspection of the BH2 region reveals very little similarity of amino acid sequence with other BH2 domains (32). In contrast, the BH2 of Bcl-G_L contains a stretch of 8 of 8 residues showing identity or conservative amino acid substitutions with the BH2 domains of other family members. By comparison, the Bad sequence reveals only 3 of 8 identical or similar amino acids in the same region. Thus, Bcl-G_L defines a novel structural variant within the Bcl-2 family of apoptosis-regulating proteins.

The production of different protein isoforms by alternative mRNA splicing is a common feature of *BCL-2* family genes, including *BCL-2, Bcl-X, MCL-1, BAX,* and *BIM* (33–37). Unlike *BCL-X,* which encodes a long and short protein, Bcl-X_L and Bcl-X_S, possessing anti-apoptotic and pro-apoptotic functions, respectively, the longer isoform of Bcl-G did not display anti-apoptotic activity. When overexpressed, Bcl-G_L induced modest and variable increases in apoptosis, whereas the shorter Bcl-G_S protein consistently exhibited potent cytotoxic activity. This behavior is reminiscent of the proteins encoded by the *BIM* gene, which include Bim-short (Bim_S), Bim-long (Bim_L), and Bim-Extra-Long (Bim_{EL}) (34). The longer proteins, Bim_L and Bim_{EL}, are sequestered in complexes with dynein light-chain

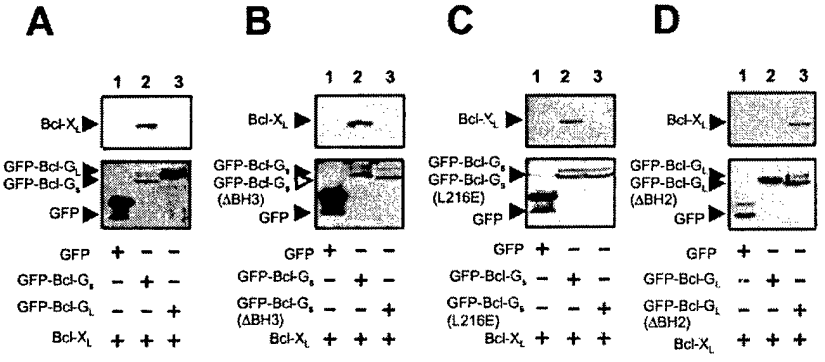

FIG. 4. **Interactions of Bcl-G_S and Bcl-G_L with Bcl-x_L.** 293T cells were transiently cotransfected with plasmids encoding Bcl-X_L and GFP, GFP·Bcl-G_L, GFP·Bcl-G_S (*A*), GFP·Bcl-G_S-ΔBH3 (*B*), GFP·Bcl-G_S-L218E (*C*), or Bcl-G_L-ΔBH2 (*D*). Cells were lysed 1 day later, and immunoprecipitations were performed using anti-GFP antibody. Immunocomplexes (prepared from 0.5 mg of lysate, *top*) and lysates (20 μg of protein, *bottom*) were subjected to SDS-polyacrylamide gel electrophoresis/immunoblot analysis using anti-Bcl-X_L (*top*) and anti-GFP (*bottom*) antibodies, respectively.

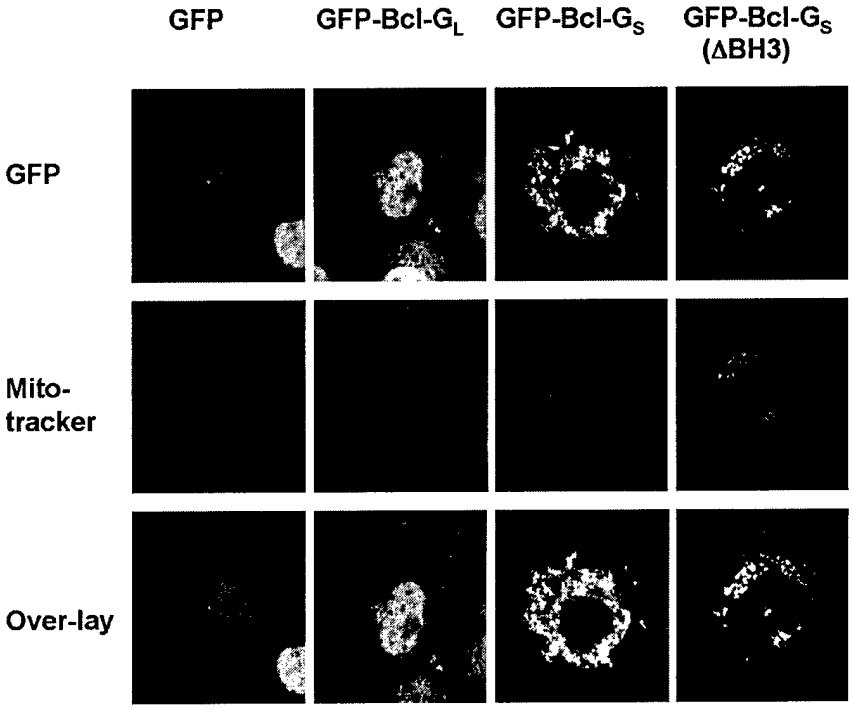

FIG. 5. **Microscopic evaluation of intracellular distributions of Bcl-G_L and Bcl-G_S.** Plasmids encoding GFP, GFP·Bcl-G_L, GFP·Bcl-G_S, and GFP·Bcl-G_S-ΔBH3 were transfected into COS-7 cells. Cells were incubated with 50 nM Mitotracker Red for 30 min, fixed in 4% formaldehyde, and examined by two-color confocal microscopy.

(DLC) in association with microtubules, thus preventing them from interacting with target proteins such as Bcl-X_L on the surface of mitochondria and other organelles (26). In contrast, because the shortest isoform, Bim_S, does not associate with DLC, it is free to interact with Bcl-X_L, Bcl-2, and other survival proteins and hence displays far more potent apoptotic activity when overexpressed in cells. By analogy, the longer Bcl-G_L protein could be sequestered in an inactive complex with an unidentified protein.

Besides interactions with sequestering proteins, the activity of pro-apoptotic Bcl-2 family proteins can be suppressed by other mechanisms, including post-translational modifications. For example, the Bad protein is inactivated by phosphorylation. This protein can be directly or indirectly phosphorylated by several protein kinases, including PKA, PKB (Akt), Raf1, and Pak1, thus preventing it from dimerizing with target proteins such as Bcl-2 and Bcl-X_L (reviewed in Refs. 30, 38). The intracellular location of Bad varies, depending on its phosphorylation state, with phosphorylated Bad residing in the cytosol and unphosphorylated Bad associated with mitochondria and other intracellular organelles where Bcl-2 and Bcl-X_L are located. In this regard, the Bcl-G_L protein contains candidate phosphorylation sites for PKA and PKC, including some not found in Bcl-G_S. However, we have been unable to demonstrate *in vivo* phosphorylation of Bcl-G_L in pilot experiments (unpublished observations).

Another post-translational modification shown previously to active latent pro-apoptotic Bcl-2 family proteins is proteolysis. Specifically, the Bid protein contains a N-terminal domain of ~58 amino acids that masks its BH3 domain, reducing its ability to dimerize with other Bcl-2 family proteins. Upon cleavage by caspases, however, removal of the N-terminal domain exposes the BH3 domain and is associated with translocation of Bid from the cytosol to mitochondria, where it induces cytochrome *c* release and apoptosis (27, 28). Whereas Bcl-G_L contains candidate caspase recognition sites, we have been unable to demonstrate significant cleavage of Bcl-G_L *in vitro* using purified active caspases or in cells during apoptosis (unpublished observations). We cannot exclude the possibility, however, that a specific caspase not yet tested is capable of cleaving and activating Bcl-G_L.

Though possessing no hydrophobic region that might anchor it in membranes, the Bcl-G_S protein was constitutively associated with intracellular organelles. Interestingly, removal of the BH3 domain did not interfere with organellar-targeting of Bcl-G_S but did abolish dimerization with Bcl-X_L. Thus, the BH3

domain apparently is not responsible for association of Bcl-G_S with intracellular organelles. This BH3-independent targeting of Bcl-G_S differs from some other BH3-only Bcl-2 family proteins such as Bad, where it has been observed that removal of the BH3 domain abrogates binding to anti-apoptotic Bcl-2 family proteins as well as association with mitochondria (39)

The *BCL-G* gene resides on chromosome 12p12, a region deleted in ~50% of prostate cancers, ~30% of ovarian cancers, and ~30% of childhood acute lymphocytic leukemias (ALLs) (40–42). Given that at least one of the protein products of the *BCL-G* gene exhibits pro-apoptotic function, it is possible that *BCL-G* represents a tumor suppressor gene. However, thus far, we have detected neither somatic mutations in the exons of *BCL-G* nor evidence of deletion of both *BCL-G* alleles in tumor cell lines or primary tumor specimens (data not shown). Further studies are required therefore to determine whether loss of *BCL-G* expression occurs in tumors by means other than somatic alterations in gene structure and DNA sequence such as changes in gene methylation or aberrant transcriptional or post-transcriptional regulation.

Investigation of the tissue distribution of Bcl-G_L and Bcl-G_S mRNAs by RT-PCR revealed that Bcl-G_L mRNA is found in several normal adult tissues, whereas Bcl-G_S was detected only in testis. This finding indicates tissue-specific regulation of Bcl-G_S mRNA splicing. Tissue-specific splicing of other Bcl-2 family mRNAs has been observed previously. For example, Bcl-X mRNA splicing events, which generate the pro-apoptotic Bcl-X_S protein occur in the thymus during T-cell ontogeny and in the mammary gland during postlactation involution, in association with extensive apoptosis induction (35, 6). Future studies therefore should include analysis of the differential mRNA splicing patterns of Bcl-G transcripts during fetal development and following various scenarios in the adult where apoptosis occurs as part of a normal physiological response or an abnormal pathological reaction to environmental insults.

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